STUDY OF RELATIONSHIP BETWEEN SUSCEPTIBILITY TO VARIOUS CANCERS AND ARYL HYDROCARBON HYDROXYLASE INDUCIBILITY

PROGRESS REPORT

September 13, 1974

Period covered: July 1, 1974 - October 31, 1974

Summary:

The core problem of this study still remains to be solved as of this date, viz. the establishment of a repeatable test. Some progress has been made in this direction, both at Dr. Kouri's laboratory and at our laboratory, but the firm, positive statement that we must be able to make before we can really begin epidemiological work, cannot yet be made.

Details of where we are in the laboratory testing here at USC are given below. In summary, the chemistry (spectrophotomer) part of the test has been satisfactorily established but the variability between split-samples of bloods run in parallel is still too great for comfort.

Dr. Kouri appears to be able to run split-samples in parallel at an acceptable level of variability, but the air-freighting of whole blood to Bethesda is not working satisfactorily (blood arriving too cold, failing to separate properly, and reduced stimulation with PHA). We have thus not yet been able to test whether day-to-day variation in

Dr. Kouri's laboratory is tight enough. (The initial collaboration between ourselves and Dr. Kouri involved cell separation, PHA stimulation, and addition of 3MC at our laboratory before sending to Bethesda for BP conversion testing: this was abandoned when it became clear that the BP conversion measurement was not the part of the assay giving trouble.)

Arrangements for collecting blood from "cases" and "controls" are set up and we envisage little or no trouble supplying the laboratories with samples once the test system is firmly established: we are currently sending 12 30 ml samples to Dr. Kouri each week testing for repeatability.

Details of Progress at USC

(a) Spectrophotomer assay for 3-Hydroxybenzo(a)pyrene (3HOBP)

This is begun by suspending the stimulated medium-free cells in one ml of a mixture of Tris buffer and $MgCl_2$, adding 100 µg benzo(a)pyrene in 50 µl acetone, and incubating at 37°C for 45-60 minutes (a blank reagent control is run without incubation). After incubation the mixture is quenched by shaking with a mixture of 1 ml acetone/ 3.25 ml n-hexane, and after centrifugation, 3 ml of the upper organic layer are withdrawn and extracted with 2 ml 1N-NaOH (aqueous). The NaOH layer is analyzed by fluorescence with an excitation wavelength of 390 nm.

We began by putting the 3HOBP through the last steps (starting with a hexane/acetone solution), using the reference material supplied by Dr. Kouri. We worked in a room under orange light (nil below 450 nm) with spectrograde solvents and high quality water, but recoveries were still erratic until we used a nitrogen purge to reduce oxidative degradation. By flushing tubes and spectrometer cuvettes and purging solvents with nitrogen we got good repeatability and linear response up to 100 p mole 3HOBP in 2 ml NaOH (see Table 1 and Figure 1: the 3 separate sets of points plotted refer to 3 different methods of 'correcting' for 'background'). Examples of the emission spectra are shown in Figure 2. Our solvents give blanks in general similar to the one shown, but there

is variation from one sample to another which would be important for samples in the range found for constitutive level AHH activities. We propose that an approximation to the solvent contribution be made by using the conventional baseline spectral correction method. In this case a line is drawn from the minimum in the 430-470 nm region tangent to the curve around 600 nm. The vertical distance between this line and the maximum at 520 nm is read as the corrected emission intensity. The example for trace 3 in Figure 2 gives a correction of .024 (= 8*.003) as against .019 (=19*.001) for correction using the solvent trace. We have also tried curvilinear corrections but these gave no improvement over the linear.

Our next step was to spike various PHA stimulated cell samples, incubated without benzo(a)pyrene, after the hexane-acetone quench, with known additions of 3HOBP. The recoveries were consistent and repeatable but not as precise as the previous series.

Further 'purely chemistry' testing appeared unwarranted by this stage as we were experiencing major biological variation.

(b) Experience with actual complete test system

Table 2 shows the stage of repeatability we have reached with the complete test system, i.e., Ficoll separation, 66 hours PHA, 24 hours 3MC, 60 minutes BP.

This sample of blood was split 10 ways -- all subsamples of 4 million lymphocytes before PHA stimulation. All subsamples had PHA added for 66 hours, then 5 had 3MC added ("induced") for 24 hours and 5 were similarly treated without 3MC ("uninduced" or "constitutive"). For each set of 5, 3 were incubated with BP for 60 minutes and 2 for zero minutes. The repeatability of these zero time controls was: for no MC, 8 and 11; for MC, 10.5 and 13.5 (scale units). Using these as corrections the constitutive levels were .084, .136, .158 and the induced levels .156, .217, .249 (arbitrary units). Using our straightline correction, this was improved to .072, .101, .112 and .143, .207, .214 giving an inducibility factor of .188/.095=1.99.

We are not satisfied with this degree of variation and are trying a number of ways to reduce it. Essentially the problem appears to be non-uniformity of PHA stimulation in separate tubes and it may be that the only solution will be to do many tubes and average the results. The quantity of blood reasonable to draw from a patient will mean that if this does turn out to be the case we will have to find micromethods of detecting 3HOBP. We will look into this possibility if necessary as soon as we have stabilized our laboratory procedures to the point where we are not reducing our variability any further. At the present time the sheer amount of manipulation required to do the test still makes it reasonable to assume that we will continue to improve for at least a few more weeks by experience alone.